Using experimental evolution to explore natural patterns between bacterial motility and resistance to bacteriophages

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Abstract

Resistance of bacteria to phages may be gained by alteration of surface proteins to which phages bind, a mechanism that is likely to be costly as these molecules typically have critical functions such as movement or nutrient uptake. To address this potential trade-off we combine a systematic study of natural bacteria and phage populations with an experimental evolution approach. We compare motility, growth rate, and susceptibility to local phages for 80 bacteria isolated from horse chestnut leaves and, contrary to expectation, find no negative association between resistance to phages and bacterial motility or growth rate. However, because correlational patterns (and their absence) are open to numerous interpretations, we test for any causal association between resistance to phages and bacterial motility using experimental evolution of a subset of bacteria in both the presence and absence of naturally associated phages. Again, we find no clear link between the acquisition of resistance and bacterial motility, suggesting that for these natural bacterial populations, phage-mediated selection is unlikely to shape bacterial motility; a key fitness trait for many bacteria in the phyllosphere. The agreement between the observed natural pattern and the experimental evolution results presented here demonstrates the power of this combined approach for testing evolutionary trade-offs.

Keywords: Coevolution/ Dispersal/ Host-parasite/ Trade-off/ Cost of Resistance

Introduction

Resistance to parasites is typically believed to be associated with fitness costs, such as reduced growth rate or competitive ability. Such costs are thought to play a key role in maintaining polymorphism in host resistance, and hence parasite persistence within populations (Antonovics & Thrall 1994; Burdon & Thrall 2003; Boots & Bowers 2004; Morgan *et al.* 2005; Morgan *et al.* 2009), and may also impact on interactions with other species both within and across trophic levels (Clancy & Price 1986; Omacini *et al.* 2001; Lennon & Martiny 2008; Hall *et al.* 2009). For example, resistance of bacteria to bacteriophages has been associated with substantial fitness costs (Lenski 1988a; Bohannan *et al.* 1999), including an increased cost of deleterious mutations (Buckling *et al.* 2006), and decreased competitive ability (Brockhurst et al. 2005; Lennon *et al.* 2007; Quance & Travisano 2009). Given the ubiquity of bacteria-phage interactions, and their key role in all ecosystems, these costs are likely to have important ecological consequences (Bohannan & Lenski 2000a; Fuhrman & Schwalbach 2003).

Here, we investigate fitness costs associated with resistance to lytic phages in the bacterial pathogen, *Pseudomonas syringae* using two complementary approaches. First, we carry out a correlational study between resistance to phages and other bacterial fitness traits (growth rate and motility) in natural plant-associated isolates. This approach allows for the direct characterization of phenotypic diversity in natural bacterial and phage populations, but can be difficult to interpret because isolates will inevitably have numerous genetic differences between them in addition to resistance to phages. Next, we test for a causal link between resistance to phages and other fitness traits using a subset of these isolates by experimentally evolving bacteria in the presence and absence of phages. This experimental evolution

approach is known to be a powerful tool for studying evolutionary trade-offs among fitness traits (Ebert 1998; Kassen 2002; Buckling *et al.* 2009), but may be less helpful in elucidating the importance of these trade-offs in explaining natural diversity. Combining these two approaches allows for a more robust assessment for the role of trade-offs in maintaining phenotypic diversity in natural populations.

Lytic phages have the potential to impose strong selection on host populations, as they are obligate killers. They replicate by injecting their viral DNA into a host bacterium, hijacking the host replication machinery to propagate, and then bursting the host cell in order to release their viral progeny (Lenski 1988b). Infection begins with the binding of molecules on the phage tail fiber to a bacterial cell surface receptor (Lindberg 1973) and resistance to phages can be gained by loss or change of these receptors, which is likely to impact on other bacterial functions (Whitchurch & Mattick 1994; Brockhurst et al. 2005). For example, phages commonly exploit bacterial surface motility appendages (flagella and pili); flagellatropic phages are known to reversibly bind to helical grooves on the bacterial flagellum and use the rotation of the flagellum to spiral towards the cell surface (Samuel *et al.* 1999), and similarly, pilus-specific phages will attach to pili and fuse their membrane with that of the bacterial cell during pili retraction (Romantschuk & Bamford 1985; Mattick 2002). A first step towards bacterial resistance may therefore be the loss or alteration of these structures. For example, phage-resistant mutants often show defective flagella that are unable to rotate (Icho & Iino 1978) and abnormal unpiliated or hyperpiliated bacteria may arise to prevent phage attachment (Bradley 1980; Mattick 2002; Brockhurst et al. 2005).

Reduced motility function is likely to have important implications for bacterial fitness in both pathogenic and non-pathogenic bacteria (Drake & Montie 1988;

Korber et al. 1994; O'Toole & Kolter 1998). Immotile mutants of the opportunistic animal pathogen *Pseudomonas aeruginosa* show reduced infectivity on human hosts and impaired biofilm formation (Drake & Montie 1988; O'Toole & Kolter 1998). Similarly, epiphytic, non-motile strains of the plant pathogen *Pseudomonas syringae* were found to have reduced fitness and competitive ability compared to more motile strains, especially under conditions of environmental stress (Haefele & Lindow 1987). Indeed, motility is a key component of fitness for bacteria in the plant phyllosphere and is necessary for successful pathogenicity, as bacteria colonizing leaf surfaces are better able to invade the leaf interior through the stomata if they are motile (Panopoulos & Schroth 1974; Beattie & Lindow 1999; Melotto *et al.* 2006).

Despite the predicted link between phage resistance and motility, and some correlative work suggesting a trade-off between the two (Joys 1965; Whitchurch & Mattick 1994), the association has never been systematically investigated. Here, we combine an examination of natural bacteria and phage isolates (from the leaves of horse chestnut trees) with experimental evolution to address this relationship. Contrary to our expectations, while natural populations of Pseudomonads show a positive relationship between bacterial motility and resistance to phages, subsequent experimental work suggests that this relationship is not clearly causal.

Materials and methods

Relationship between resistance and motility in natural populations

To investigate the natural variation in resistance to phage and motility, we examined 80 natural isolates, from either the surface or the interior of horse chestnut leaves collected around Oxfordshire, United Kingdom, that were part of a larger sampling design from a previous experiment (Koskella et al. 2011). The study included a reciprocal cross-inoculation of culturable bacteria and communities of

phages isolated from each of 32 leaves. Bacteria were isolated from either the leaf surface, using buffer from leaf washes, or leaf interior, using homogenates from surface-sterilized leaves. Washes/homogenates were plated on 1.2% King's medium B (KB) broth (10 g/litre glycerol, 20 g/L proteose peptone #3 (Becton Dickinson UK Ltd), 12 g/L agar, 1.5 g/L K_2HPO_4•$3H_20$, 1.5 g/L $MgSO_4$•$7H_20$) and, after 48 hours of growth, colonies were picked at random based on proximity to a randomly chosen spot on the plate. Phages from the interior and surface of each leaf were separated from bacteria by chloroform treatment of the buffer solutions. This allowed us to generate an inoculum that was representative of the natural phage community as it did not require passaging through a bacterial host. Using a cross-inoculation design, 7 µl of each phage inoculum was spotted in a grid formation onto a lawn, grown in soft KB agar (0.6% wt/vol), of each bacterial isolate. Phage plaque formation within the spot was compared with bacterial growth across the lawn, allowing us to define each bacterial isolate as either susceptible or resistant to the local, sympatric phage population (*i.e.,* to quantify susceptibility to phages from the same leaf). We then randomly chose 40 bacterial isolates from each category (susceptible or resistant) to further characterize and measure motility and growth rate. Of the 80 isolates examined, only 6 pairs showed strong phenotypic and genotypic similarity, and each of these pairs was combined in our statistical analyses.

Bacteria typically exhibit three types of motility: swimming, swarming, which are primarily flagella-dependent, and twitching, which is dependent on type IV pili (Mattick 2002; Harshey 2003). To examine these motility mechanisms, we measured dispersal capability of each of the bacterial isolates under different agar environments. For each motility assay, bacterial isolates were first grown overnight from freezer stocks in KB broth at 28 ˚C. A small amount of each culture was then

used to inoculate the center of 90 cm^2 petri dishes containing 25 mL of KB medium with the appropriate concentration of agar, dried briefly before use. Twitching motility was assessed on KB medium solidified with 1.2% (wt/vol) agar and estimated using the bacterial movement between the interface of the petri dish and agar surface, while swarming and swimming motility assays were performed on KB medium containing 0.6% and 0.3% (wt/vol) agar, respectively (Rashid & Kornberg 2000), and estimated by area dispersed through the agar. The area of dispersal was measured after either 24 hours (for swimming) or 48 hours (for swarming and twitching) of incubation at 24 ˚C by demarcating the area covered, photographing the plate with a measurement standard, and analyzing the area digitally using ImageJ 1.41o (Abramoff *et al.* 2004). All area data were square root transformed and three replicate assays were run for each bacterial isolate.

In vitro *growth rate and density assays*

We measured the growth rate and final bacterial density for each of the 80 natural bacterial isolates. For growth rate assays, KB cultures were grown overnight at 28˚C and diluted by a factor of 1:100. Then, 10 µl of each dilution was added to a 96-well microplate containing 90 µL of KB per well and optical density at 600 nm was measured every 45 minutes at an incubation temperature of 24 ^{o}C with 5 second shaking prior to read for 24 hours using a microplate spectrophotometer (BioTek Powerwave XS, Northstar Scientific Ltd., Bedfordshire, UK). The period of exponential growth occurred between 4 and 12 hours, during which time V_{max} (measured as milli-optical density units per minute (mOD/ min)), the maximal rate of change in optical density during log growth, was calculated. For density assays, cultures incubated for 24 hours were diluted by a factor of 1:1 (in order to bring them

within a range of measurements for which the readings were most accurate) and measured for optical density at 600 nm.

Characterization of isolates

The identity of each bacterial isolate, to the genus level, was determined by sequencing an 800 bp region of the 16S rRNA subunit using the forward primer 27F (Lane 1991) and reverse primer 907R (Muyzer *et al.* 1998). The reaction contained 1 U *Taq* DNA polymerase (Invitrogen, Paisley, UK), 1X *Taq* Buffer, 3 mM $MgCl_2$, 0.2 μM dNTP's, 0.2 pm of each primer, and 0.5 μL of a 1:10 dilution of an overnight KB culture. PCR amplification was performed at 95 °C for 4 minutes, 29 cycles of 95 °C for 45 seconds, 52 °C for 1 minute, and 72 °C for 2 minutes, with a final elongation at 72 °C for 10 minutes. The product was then sequenced by Geneservice (Oxford, UK) using the reverse primer. These sequence data have been submitted to the GenBank database under accession Numbers HQ529384-HQ529465. Each bacterial isolate was assigned to the genus level, using the NCBI database, based on highest sequence similarity; all but five of the isolates had over a 97% similarity to a previously characterized isolate, with the other five being between 93 and 95% similar and all isolates had an e value of 0. Isolates were not assigned to the species level due to the highly conserved nature of the sequenced 16S rRNA region.

Selection Experiment

To specifically examine how the acquisition of phage resistance might alter motility function we performed an experimental evolution study using a random subset of the natural isolates. Because of both the observed positive correlation between motility and resistance to phages and the epidemiological significance of the species (Hirano & Upper 2000; Webber *et al.* 2008; Green *et al.* 2009), we chose to

focus exclusively on 10 bacterial isolates from the leaf interior that had >99% sequence similarity to known isolates of *Pseudomonas syringae.* Importantly, these isolates were all sampled from separate leaves to decrease the probability of pseudo-replication. To experimentally examine the relationship between swarming motility and phages in the environment, we performed 10 serial transfers (approximately 10-16 bacterial generations per transfer) of each bacterial isolate into fresh soft agar (0.6% wt/vol) that contained either high concentrations, low concentrations or no phages (Figure 1). To initiate the experiment, overnight cultures from a single bacterial colony were grown in KB broth, which was then pipetted directly onto the centre of a 144 cm^2 square petri-dish (Fisherbrand, Leicestershire, UK) containing 40 ml soft KB agar. Plates were left in a humid incubator at 24 ^oC for 24 hours, after which samples were taken for the next transfer under one of two selection regimes: positive (termed 'dispersal' treatment) or neutral (termed 'random' treatment) selection for dispersal (Figure 1). For the dispersal selection lines, we took six samples, equally spaced apart, from the outer edge of the colony range. For the random selection bacterial lines we took six samples from throughout the colony, as chosen by a random number grid. Each sample was taken by stabbing a sterile 1 ml pipette (Finnpippette, Northumberland, UK) through the agar to the bottom of the plate and then transferring the agar stab to 1 ml of M9 solution (1 mM thiamine hydrochloride, 0.4% glycerol, 0.2% casamino acids, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$). The pipette tip was washed thoroughly in the solution and, after vortexing, 2.5 µl of the solution was used to inoculate fresh agar plates, as described above. This was repeated every 24 hours for ten transfers. Plates were poured with fresh media at each transfer so as not to confound age of agar plate with dispersal ability.

The phage inoculum was generated by isolating 16 individual phage types (*i.e.,* independent plaques) from 16 different horse chestnut leaves used in the previous cross-inoculation (Koskella et al. 2011). Each phage isolate was passaged once through one of two previously characterized strains of *P. syringae pv. aesculi* (*P.s. pv aesculi* 6617 and 6623; Green *et al.* 2010), in order to amplify numbers of phage particles, and separated from the bacteria using chloroform. The 16 phage isolates were mixed to produce a stock inoculum, representing a subset of the naturally occurring phage community in the leaf environment, and stored at 4 °C. This design allowed us to hold the phage environment relatively constant while the bacteria evolved in response. To create a homogeneous selective environment, phage inoculum was vortexed into the soft agar prior to solidifying, when the agar reached about 40 °C. After ten transfers, each line was assayed for growth rate and motility in a phage-free common garden (*i.e.,* all treatments were grown under the same laboratory environment), and under three agar concentrations. Bacterial densities (OD_{600}) of overnight cultures of each evolved and ancestral strain were also measured in both the presence and absence of phages.

Statistical analyses comparing bacterial dispersal and susceptibility to phages

Analyses and figures were produced on PASW Statistics 18 (SPSS; part of IBM UK ltd, Middlesex, UK). We first used a two-way analysis of variance to compare the area dispersed (square-root transformed) across bacterial isolates of different genera and susceptibility to phages. In addition, independent samples t-tests were run to compare susceptibility to phages and dispersal within the *Pseudomonas* and *Erwinia* isolates from both the leaf surface and leaf interior. For the experimental evolution results, we examined the initially susceptible and initially resistant isolates separately, due to the dramatic differences in means and variance between them,

and we included bacterial strain as a random factor in each model. At the end of the experiment, we examined the area dispersed within a common garden (in the absence of phages) at the end of the experiment using a separate two-way analysis of variance for (1) the 30 susceptible lines and (2) the 30 resistant lines and compared population growth parameters to dispersal using Pearson correlation coefficients. In all cases, the area dispersed was square root transformed.

Results

Relationship between resistance, growth and motility in natural populations

Sequencing of the chosen bacterial isolates revealed that the surface of the leaf was primarily dominated by *Erwinia*-like species (>85%), while the leaf interior community was comprised of both *Erwinia*-like (50%) and *Pseudomonas*-like species (47%). We excluded bacterial isolates from other genera, including *Rhanella* and *Pantoea,* from subsequent analyses due to low replication. Overall, 44% (N/N_{TOT}=24/54) of the *Erwinia*-like isolates and 61% (N/N_{TOT}=14/23) of *Pseudomonas*-like isolates were susceptible to sympatric phages, i.e. those collected directly from the same leaf as the bacteria being tested. We chose to focus specifically on susceptibility to sympatric phages, using the leaf homogenate as an inoculum, because this measure more accurately reflects local selection pressures and did not require amplification through a bacterial host.

The relationship between swarming motility and resistance to sympatric phages significantly differed across bacterial genera (Figure 2a; interaction effect for genus x phage susceptibility: $F_{1,38}$= 5.99, P = 0.020). Specifically, there was no difference in dispersal capability between resistant and susceptible strains of the *Erwinia*-like isolates from the leaf surface or interior (Figure 2a). However, for the *Pseudomonas*-like isolates from the leaf interior (surface isolates were excluded due

to low sample size) resistant isolates showed a higher swarming motility range than the susceptible isolates (Figure 2a; t_{17}= 2.241, P= 0.039) but did not show a difference for swimming or twitching motilities (swimming: t_{17}= 1.143, P= 0.271; twitching: t_{17}= 1.219, P= 0.099).

There was no difference in growth rate, measured as V_{max} (mOD/ min) between resistant and susceptible *Erwinia*-like isolates from the surface or the interior (P > 0.05), but for *Pseudomonas*-like isolates from the leaf interior, resistant isolates had a higher growth rate than susceptible isolates (Figure 2b; t_{17}= 3.234, P= 0.005). Importantly, the dispersal area during swimming and swarming motility was significantly correlated with growth rate for both the *Pseudomonas*-like isolates (Pearson correlation, swimming: r= 0.517, P= 0.012; swarming: r= 0.669, P< 0.001) and the *Erwinia*-like isolates (swimming; r= 0.317, P= 0.019; swarming: r= 0.359, P= 0.008). However, twitching dispersal was not correlated with growth rate for either *Pseudomonas*-like isolates (r= 0.310, P= 0.150) or *Erwinia*-like isolates (r= -0.100, P= 0.473).

Selection for bacterial resistance to phages and/or motility

Of the ten Pseudomonad isolates chosen for experimental evolution, five were initially susceptible and five were initially resistant to the phage inoculum. Of the initially susceptible strains, all were susceptible to at least half of the 16 phage isolates used in the inoculum (mean susceptibility of 70.0% ±19.7 SD). Of the five initially resistant bacterial isolates, resistance was complete across all 16 phage isolates (susceptibility of 0%). At the start of the experiment the presence of phage had a significant negative effect on motility for the initially susceptible strains ($F_{1,24}$= 7.223, P= 0.013), but had no effect on the initially resistant strains ($F_{1,24}$= 1.624, P=

0.215). After ten transfers of experimental evolution in the presence of phages (at both high and low concentration and across random and dispersal selection regimes), each of the five bacterial isolates that were initially susceptible to phages had evolved resistance to most or all of the 16 phage isolates used in the inoculum (Figure 3). For these isolates there was no longer an effect of phages in the environment on motility ($F_{1,24}$= 0.400, P= 0.533). These strains did not evolve increased resistance in the absence of phages (GLM for mean proportion of infective phages, effect of Time: $F_{1,18}$= 16.56, P= 0.002; Phage treatment: $F_{2,18}$= 4.173, P= 0.032; Time x Phage treatment interaction: $F_{2,18}$= 4.173, P= 0.032). The bacterial isolates that were initially resistant remained resistant throughout the duration of the experiment. Although we did not directly allow for coevolution between bacteria and phages (Bohannan & Lenski 2000a; Buckling et al. 2009), some degree of coevolution may have occurred between phages that were passively collected along with bacteria at each transfer; creating an additional benefit to dispersing away from the inoculation site, as coevolved phages are likely to have increased infectivity to the evolving bacteria (Bohannan & Lenski 2000a; Brockhurst et al. 2005). However, all resistance assays were run using ancestral phages, as these represented the selection pressure throughout the experimental environment.

To confirm our measures of phage resistance, we also measured growth rates of ancestral and evolved lines in the presence of phages. For those strains that were initially resistant, we found no difference in population density in either the presence or absence of phages (GLM with arcsinsqrt transformed density, interaction effect of Time x Phage presence: $F_{1, 42}$= 0.206, P= 0.652). However, for those strains that were initially susceptible and evolved resistance to phages over the course of the experiment, the ancestral bacterial lines had a significantly lower density than

evolved bacterial lines in the presence of phages, but not in the absence of phages (Time x Phage presence: $F_{1, 42}$= 5.977, P= 0.019), indicating that phages were initially reducing population size of susceptible bacteria but that population size was not affected by phages once resistance had evolved.

We measured area dispersed and growth rate of all isolates at the start of the experiment in the absence of phages and found that the initially resistant bacterial isolates had a higher mean swarming dispersal (mean area dispersed 56.45 $cm^2 \pm$ 7.05 SD in 0.6% agar) than the susceptible isolates (3.14 $cm^2 \pm$ 3.36 SD) and that initially resistant isolates had a higher growth rate (mean Vmax 3.49 $\pm$ 0.36 SD) than susceptible isolates (1.55 $\pm$ 0.79 SD). These results were consistent with the findings from the full sample of natural bacterial isolates.

After the ten serial transfers of experimental evolution we again assayed motility in a phage-free environment to examine the effect of both phage and dispersal selection regime on the evolution of dispersal, without the confounding ecological effects of phages (Figure 4). The initially susceptible strains did not show a response to selection for increased dispersal ($F_{1,20}$= 0.333, P= 0.571) and there was no evidence for a direct effect of phage treatment on dispersal ($F_{2,20}$= 0.337, P= 0.718) nor for any interaction between phage and selection ($F_{2,20}$= 0.080, P= 0.923), suggesting a lack of a causal link between phage resistance and motility (Figure 4a). However, the initially resistant strains were able to respond to selection for increased dispersal ($F_{1,20}$= 10.505, P= 0.004), regardless of phage treatment (main effect of Phage: $F_{2,20}$= 1.995, P= 0.162, interaction between Phage and Selection: $F_{2,20}$ = 0.514, P= 0.606, Figure 4b). Finally, there were few correlated changes in swimming or twitching motility resulting from either the dispersal or phage selection regimes: the only significant effect, after controlling for multiple tests, was increased swimming

motility under positive selection for dispersal (compared with random) in the initially resistant lines (Figure 4b; main effect of selection: $F_{1,20}$= 8.268, P= 0.009).

We also investigated how the selection regimes affected growth rate of each population, with the specific focus on whether there were costs associated with the acquisition of resistance. We found no evidence that dispersal selection regime or phage-imposed selection affected population growth rate for either initially resistant or initially susceptible bacteria (P> 0.10 for all treatments). Importantly, although we found a correlation between growth rate and dispersal for the initially resistant strains (Pearson r= 0.390, P= 0.033) there was no correlation between growth rate and dispersal for the initially susceptible lines (r= 0.000, P= 0.999), demonstrating that dispersal estimates were not simply a function of population growth. This result was qualitatively the same when comparing population density and dispersal.

Discussion

We combined an examination of natural phenotypic variation with experimental evolution to investigate the potential link between resistance to phages and bacterial fitness (growth rate and motility). We found that, contrary to expectation, natural bacterial isolates that were resistant to local phages had neither reduced motility nor reduced growth rates relative to those that were susceptible. Generally, there was no relationship between bacterial motility or growth rate and resistance to phages from the local environment (as present in the leaf homogenate) for *Erwinia*-like isolates and a positive correlation for *Pseudomonas*-like isolates under favorable, laboratory conditions (Figure 2). This result is in line with previous work from marine Cyanobacteria showing that costs of resistance to phage are not ubiquitous and instead, are highly dependent on the virus and bacteria strain being

examined and on whether strains are in direct competition (Lennon *et al.* 2007). Importantly, we could not rule out the possibility that more motile and resistant strains represented different species or pathovars than less motile and susceptible strains. Therefore to investigate any causal links between resistance to phages and bacterial motility, we experimentally evolved 10 *Pseudomonas*-like bacterial lines in either the presence or absence of phages, and under either positive or random selection for dispersal.

After ten serial transfers of experimental evolution we found that bacteria that were initially susceptible to phages had evolved resistance in the presence, but not the absence, of phages (Figure 3). Moreover, at the start of the experiment we saw decreased motility in the presence of phages for those strains that were susceptible to infection, but not for those that were initially resistant. This result adds to a growing body of evidence that parasites can have a direct impact on host demography (Fellous et al. 2010; Bradley et al. 2005; Cameron et al. 1993). In addition, we found that bacterial motility decreased over the course of the experiment for most bacterial isolates (regardless of phage treatment and even under selection for increased dispersal), suggesting a cost to motility such that flagella and/or pili function is reduced under favorable laboratory conditions. Importantly, the fact that the initially resistant bacteria showed reduced motility but no loss of resistance to phages in the control (no-phage) treatments, suggests that there is no negative correlation between resistance and either growth rate or motility in the laboratory. This overall reduction in motility is initially surprising given that half the lines were selected for increased dispersal and suggests that the imposed selection regime was relatively weak, as only the initially resistant, and not the initially susceptible, lines showed increased motility under selection for high dispersal (Figure 4). This result warrants further study

as we cannot provide a clear mechanistic explanation, and understanding whether phages directly hinder the response of susceptible bacteria to selection for increased motility is of key interest. In addition, under the no-phage, common garden conditions at the end of the experiment, we found no evidence that phage-imposed selection for resistance in the initially susceptible lines was associated with a change in either the motility trait under selection (swarming), or twitching and swimming motility (Figure 4). Finally, there was no evidence of growth costs associated with the acquisition of resistance (although the strains were never put in direct competition). These data suggest no causal relationship, either positive or negative, between the acquisition of phage resistance and dispersal ability.

A number of other studies, like ours, have found no clear cost in terms of population growth associated with resistance to phages (Lenski 1988a; Lythgoe & Chao 2003; Meyer et al. 2010). However, the lack of an observed pattern is somewhat surprising given the predicted association of many phages with flagella or pili number and function (Icho & Iino 1978; Bradley 1980; Mattick 2002). There are a number of possible explanations. First, phages that use motility organelles (*i.e*., pili and flagella) as attachment sites may be relatively uncommon in the natural leaf environment and hence do not impose very strong selection against bacterial motility. This interpretation may have been influenced by our phage isolation method; chloroform treatment is known to destroy primarily lipid-based phages (Leers 1969), and if a correlation exists between phage-targeted bacterial receptors and phage coat composition we could have missed an effect of phage-mediated selection. Second, observed dispersal behaviors may be influenced by traits other than motility organelles, such as cell size, chemotaxis or quorum sensing (Harshey 2003), which are not altered by the acquisition of phage resistance; a possibility that would indicate

bacterial motility can evolve independently of phage-mediated selection. Third, it is plausible that motility-associated costs may only be visible under stressful environmental conditions. For example, previous experimental work has shown that costs of resistance to phages in both *Escherichia coli* B (Bohannan & Lenski 2000b) and *Pseudomonas fluorescens* (Lopez-Pascua & Buckling 2008) are increased in nutrient-poor environments. Fourth, it is possible that any costs were rapidly compensated by second site mutations, as is commonly observed with antibiotic resistance (MacLean *et al.* 2010). However, such rapid compensation would suggest that costs of phage resistance are relatively unimportant in natural populations.

Overall, our results do not support a causal link between the evolution of resistance to phages and bacterial motility in natural populations, although we did find a surprising positive association between phage resistance and bacterial motility for Pseudomonads in the phyllosphere. This of course does not rule out an important role of phage-imposed selection on the evolution of motility, as costs associated with resistance are likely to be contingent upon genetic background, local environment and the precise measures of fitness, but does suggest that the effect of phage-imposed selection is unpredictable and is likely to depend on the natural phage community and bacterial environment. This is an important consideration for phage therapy of pathogenic bacteria, where virulence is often associated with motility traits and any association between phage resistance and bacterial motility could have important consequences over coevolutionary time (Josenhans & Suerbaum 2002). Understanding these potential costs will be key as phage therapy becomes a more common method for controlling pathogenic bacterial populations (Goodridge 2004; Levin & Bull 2004). More generally, these results add to a growing body of work across a range of taxa that points to the wide range of host fitness traits correlated

with pathogen resistance (Boots & Begon 1993; Ferdig et al. 1993; Fellowes et al. 1998; Langand et al. 1998; Zhong et al. 2005; Buckling et al. 2006; Morgan et al. 2009; Williams et al. 1999; Yourth et al. 2002; Lythgoe & Chao 2003; Sanders et al. 2005). This method of combining natural observations with laboratory selection experiments shows promise in furthering our understanding of the importance of phages, and other selective pressures, as drivers of bacterial evolution and diversity in natural environments.

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Figure 1. Ten natural isolates were experimentally evolved over ten transfers in one of three phage environments: high phage concentration (40 µl phage inoculum /40 ml 0.6 % agar), low phage concentration (4 µl phage inoculum /40 ml 0.6 % agar) or no phage treatment (where no phage was added to 0.6 % agar). In addition, each line was evolved under one of two selection regimes: positive (termed 'dispersal' treatment) or neutral (termed 'random' treatment) selection for dispersal. In total, we had 60 selection lines evolved over approximately 140 generations of selection.

Figure 2. Relationship between dispersal (a) and growth rate (b) for the 40 *Pseudomonas*-like and *Erwinia*-like isolates collected from the leaf interior. Resistance (dark grey) or susceptibility (light grey) to local phage is compared to determine whether there exists a cost to resistance with regard to bacterial motility or growth. Dispersal was measured as area covered (cm^2) over 48 hours on soft agar (0.6%). Values were square root transformed to correct for non-normality. Error bars represent ±1 SEM.

Figure 3. Evolution of resistance for the five initially susceptible isolates over ten serial transfers in either the presence (dashed lines) or absence (solid line) of phage. Proportion of infective phage represents susceptibility to each of the 16 phage clones used in the experimental evolution inoculum. Error bars represent ±1 SEM.

Figure 4. Results from common garden experiment run at the end of the selection experiment (*i.e.,* transfer 10) for initially resistant (a) and initially susceptible (b) isolates. Measured on 0.3 % agar (swimming; left panel), 0.6 % agar (swarming; middle panel); and 1.2 % agar (twitching; right panel). These experiments were run in

the absence of phage in the environment to examine differences between the lines that are not resulting from interactions with or ecological feedback from phage. "Treatment" therefore represents the phage environment of each line during the course of experimental evolution and not within the common garden experiment. Area dispersed is square root transformed and error bars represent ±1 SEM.

Figure 1.

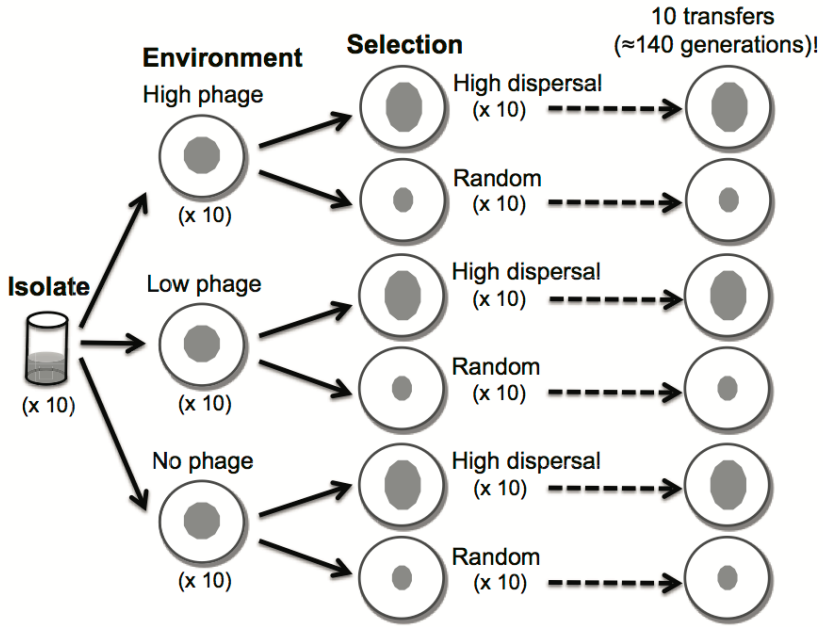
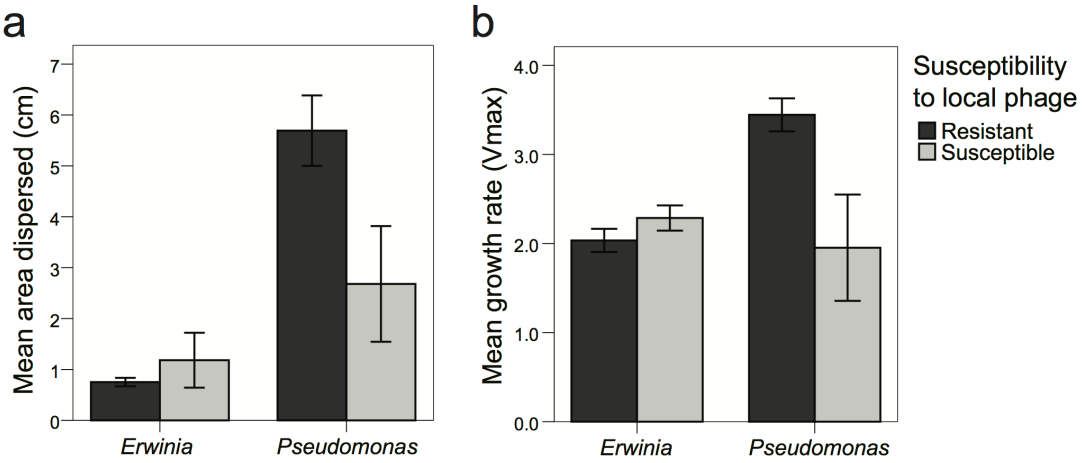
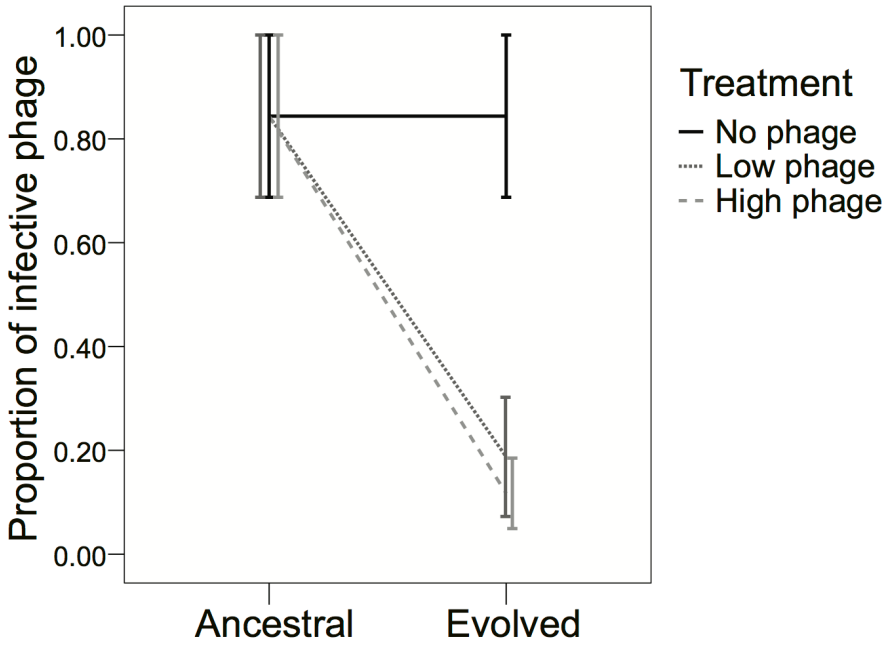

Environment
Selection
10 transfers
(≈140 generations)!
High phage
(x 10)
High dispersal
(x 10)
Random
(x 10)
Isolate
(x 10)
Low phage
(x 10)
High dispersal
(x 10)
Random
(x 10)
No phage
(x 10)
High dispersal
(x 10)
Random
(x 10)

Figure 2.

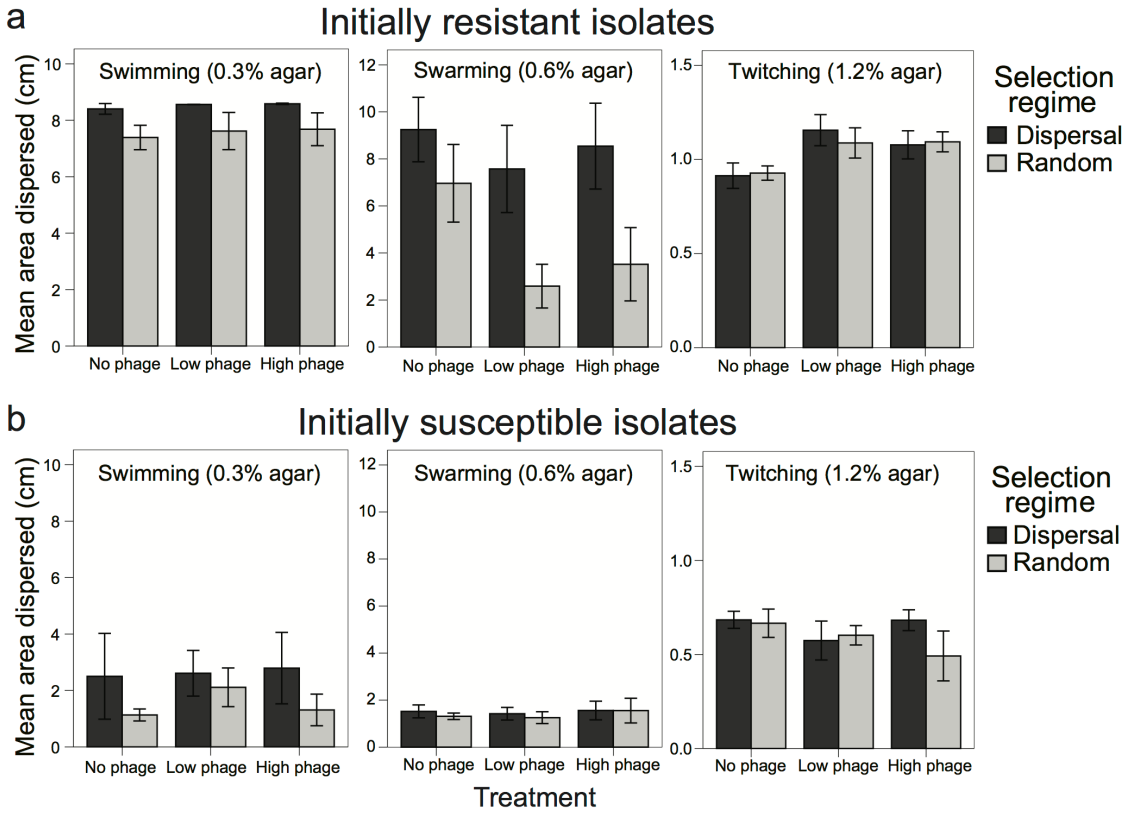

a
Mean area dispersed (cm)
0
1
2
3
4
5
6
7
Erwinia
Pseudomonas
b
Mean growth rate (Vmax)
0.0
1.0
2.0
3.0
4.0
Erwinia
Pseudomonas
Susceptibility to local phage
Resistant
Susceptible

630

Figure 3.


Proportion of infective phage
1.00
0.80
0.60
0.40
0.20
0.00
Ancestral
Evolved
Treatment
No phage
Low phage
High phage

635 Figure 4.


a
Initially resistant isolates
Swimming (0.3% agar)
Swarming (0.6% agar)
Twitching (1.2% agar)
Mean area dispersed (cm)
No phage
Low phage
High phage
Selection regime
Dispersal
Random
b
Initially susceptible isolates
Swimming (0.3% agar)
Swarming (0.6% agar)
Twitching (1.2% agar)
Mean area dispersed (cm)
No phage
Low phage
High phage
Treatment
Selection regime
Dispersal
Random